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PRINCIPAL INVESTIGATOR: Rudolf J. Kaaks, Ph.D.

CONTRACTING ORGANIZATION: International Agency for Research on Cancer  
69372 Lyon, Cedex 08 France

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14. ABSTRACT <p>Purpose and scope: we are conducting a large case-control study, nested within the Dutch DOM prospective cohort, to estimate relative risk of breast cancer in post-menopausal women by levels of urinary estrogens and estrogen metabolites.</p> <p>Progress report: in this year of extension of the project, we finalized the setting up of our sensitive NCI GC/MS method for the measurement of estrogen metabolites in urine samples. By using this method, we performed these measurements on 145 breast cancer cases and 145 matched controls.</p> <p>Conclusions: On the first set of data, concentrations of 2-hydroxy estrone, 2-methoxy estrone and the ratio between 2-hydroxy estrone and 16-alpha hydroxy estrone were significantly associated with breast cancer risk in post-menopausal women. We plan to complete the analyses on the samples from the whole nested case-control study by April 2007 (i.e., beyond the extension limits of this grant, and using internal IARC funds). A manuscript will then be prepared, reporting the relationships of the urinary hormone levels with breast cancer risk.</p>					
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## INTRODUCTION:

It has long been recognized that estrogenic steroid hormones, particularly 17  $\beta$ -estradiol ( $E_2$ ) can promote the development of breast tumors. Besides stimulating cell proliferation, there is increasing experimental evidence that estrogens may also be activated into genotoxic hydroxy metabolites that cause DNA mutations. In addition, some of the same metabolites may bind irreversibly to estrogen receptors, and thus stimulate cell proliferation permanently.

Major pathways through which hydroxy metabolites of estrogens (estrone [ $E_1$ ] and estradiol [ $E_2$ ]) are formed are the 16 $\alpha$ -hydroxylation pathway – which leads to formation of 16 $\alpha$ -hydroxy  $E_1$  and estriol – and pathways that lead to 2- and 4-hydroxy (“catechol”) estrogens. Preliminary epidemiological evidence suggests that estrogen metabolism via the 16 $\alpha$ -hydroxy pathway is increased in breast cancer patients compared to controls, and an inverse relationship has been found between breast cancer risk and the ratio of urinary concentrations of 2-hydroxy/4-hydroxy or 2-methoxy/4-methoxy estrogens.

Amongst key enzymes involved in the natural conversion of estrogens to hydroxy estrogens are CYP1A1, CYP1B1, and CYP3A4. Furthermore, catechol-O-methyl transferase (COMT) is a key enzyme in the methoxylation of 2- and 4- hydroxyl groups, thus leading to methoxy estrogens. Methoxylation is a major pathway for the inactivation of the chemically very reactive catechol estrogens. In addition, experimental studies indicate that the methoxy metabolites inhibit tumor formation and development by decreasing cell growth, and inhibiting the formation of blood vessels in tumors.

Given these various observations, it has been hypothesized that breast cancer risk would be lower in women who produce more 2- and 4-methoxy estrogens relative to the levels of the corresponding hydroxyl estrogens. To examine the above hypotheses, we have started a case-control study nested within a large prospective cohort (the ‘DOM’ cohort, the Netherlands), with the following specific aims:

- 1) examine relationships of post-menopausal breast cancer risk with absolute and relative prediagnostic urine levels of 2-hydroxy, 4-hydroxy, 16 $\alpha$ -hydroxy, 2-methoxy and 4-methoxy metabolites of  $E_1$  and  $E_2$
- 2) examine relationship of polymorphic variants of genes encoding estrogen-metabolizing enzymes (CYP1A1, CYP1B1, CYP3A4 and COMT) to urinary levels of the various estrogen metabolites, as well as to breast cancer risk.

Our project is designed as a case-control study nested within a large prospective cohort, using urine and DNA samples collected from more than 50,000 women in the Dutch city of Utrecht and

surroundings (“DOM” cohort). This cohort is unique, in that rather large volumes (50-100 ml) of urine were collected and stored for all study subjects. The majority of women in the cohort provided also a second (and even third) urine sample. The samples were stored in a large frozen warehouse. Relatively large volumes of urine (>10 ml) are needed to measure the estrogen metabolites, by gas chromatography coupled with mass spectrometry (GCMS). Cases and controls are selected among women who were post-menopausal at recruitment, and who did not use hormone replacement therapy. For about 60% of women who provided a second urine sample within a time interval of about one year we also incorporated this second sample in our study, so as to improve exposure measurements.

## **BODY:**

The statement of work for this project included a total of seven tasks:

**Task 1.        *Selection of cases and controls, using the established eligibility and matching criteria, and extraction of case-control data sets with relevant information from questionnaires and anthropometry.***

This task was completed during year 1 of the project. A total of 324 cases and 324 matched controls, were selected from the DOM cohort population

**Task 2.        *Retrieval of urine samples (1<sup>st</sup>, 2<sup>nd</sup> and 3<sup>d</sup> visits) from the “DOM” repository; centrifugation of urines to prepare pellets containing cells with DNA (for extraction); assembly of the urine samples into batches of matched case-control sets for analysis of urinary estrogen metabolites.***

This task was also completed during year 1 of the project. For all 324 cases and 324 matched controls, urine samples retrieved and shipped to the laboratory of Dr. Kurzer, University of Minnesota, for analyses of estrogens and estrogen metabolites, as originally planned.

**Task 3.        *Measurement of urinary levels of estrogen metabolites by GC-MS, for all 300 cases and 300 controls.***

This task has been only partially completed, due to serious initial problems with the hormone assays. Unfortunately, after a short initial delay due to budget transfer, a substantial delay in the work occurred in Dr. Kurzer’s laboratory because of internal re-organization of personnel. The postdoc and a laboratory technician that were in charge of the estrogen metabolite measurements in Dr Kurzer’s laboratory left, and needed to be replaced by new personnel. Using the funds of her

subcontract, Dr Kurzer hired a new technician, who finally started in June 2003 to work full-time on the estrogen metabolite measurements for our project. In September 2003 this technician had been fully trained to run the GCMS method measurements, and in the meantime Dr. Kurzer hired a second laboratory technician so as to accelerate the pace of measurements and recuperate the time lost during last several months of year 1.

Unfortunately, however, in the Year 2 of the project, important problems of sensitivity in the estrogen metabolites measurements were encountered in Dr. Kurzer's laboratory, and again substantial delay has been cumulated. After measuring (and repeated re-measuring) about 30 samples, the laboratory analyses were stopped. In those first samples, only estrone and 2-methoxyestradiol were detected and measured in all subjects, while estriol and 2-hydroxyestrone were detected in 80% of the subjects, 2-methoxyestrone and 2-hydroxyestradiol were detected in 50% of the subjects, 4-hydroxy estrone, 16-alfa hydroxyl estrone and 4-hydroxy estradiol were measured in less than 30% of the subjects, and estradiol, 4-methoxyestradiol and 4methoxyestrone were not detected at all in any of the subjects. After some tests (for detail, please see Progress Report of Year 2), we decided that the major problem with the estrogen metabolite measurements was not the degradation over time of the metabolites in the urine samples, but a lack of sensitivity of the method used for the assays (the detection limit was finally determined to be about 50ng/ml).

We therefore decided to perform these assays by using a different method, much more sensitive than the method used by Dr. Kurzer (about 100 times more), that was being set at the laboratory of the Hormones Team at the International Agency for Research on Cancer (IARC) for measurements of estrogen metabolites in blood. Before applying it to urine samples, the method had to be converted for the application to urine samples, instead of blood serum.

The urine samples from the DOM cohort were subsequently shipped back from the University of Minnesota to our laboratory at IARC, between November 2005 and January 2006. During the Year 2005, we eventually finalized the set-up of a very sensitive method for the analyses of estrogen metabolites in both blood and urine samples, based on negative chemical ionization gas chromatography/mass spectrometry (GC/ NCI-MS). Our method is based on enzymatic hydrolysis, solid phase extraction, purification by high performance liquid chromatography (HPLC), derivatization with fluorinated agents (essential to have a very sensitive detection by NCI-MS) and final injection on GC/ NCI-MS. The detection limits for each of the hormones are the following:

E <sub>2</sub>	50 pg/ml
E <sub>3</sub>	50 pg/ml

16 $\alpha$ OH-E <sub>1</sub>	50 pg/ml
2OH-E <sub>2</sub>	50 pg/ml
4OH-E <sub>2</sub>	50 pg/ml
2OMe-E <sub>2</sub>	125 pg/ml
4OMe-E <sub>2</sub>	500 pg/ml
E <sub>1</sub>	500 pg/ml
2OH-E <sub>1</sub>	500 pg/ml
4OH-E <sub>1</sub>	500 pg/ml
2OMe-E <sub>1</sub>	125 pg/ml
4OMe-E <sub>1</sub>	250 pg/ml

The finalization of the method at IARC had some degree of delay because of problems of stability of the GC/MS. Continuous shifting of the recorded masses could not allow a proper determination of the estrogen peaks. After several tests, all indicating a problem with the block controlling the radiofrequency, we decided to completely change the block. After this change, the GC/MS got back to its normal stability, and measurements could be continued.

For a first series of 130 urine samples we performed our analyses in duplicate, to check the robustness of the method, and to determine intra-class correlations between the duplicate (see Table 1).

**Table 1. Reproducibility of estrogens and estrogen metabolite measurements by NCI GC/MS (Intra-class correlations-ICC) on 130 urine samples from the DOM cohort.**

<i>Hormone</i>	<i>ICC (Confidence interval)</i>
Estrone	0.91 (0.89-0.98)
Estradiol	0.91 (0.84-0.97)
Estriol	0.96 (0.94-0.99)
16- $\alpha$ hydroxy estrone	0.87 (0.79-0.95)
2-hydroxy estrone	0.89 (0.83-0.97)
2-hydroxy estradiol	0.90 (0.84-0.96)
4-hydroxy estrone	0.85 (0.76-0.97)
4-hydroxy estradiol	0.98 (0.97-0.99)
2-methoxy estrone	0.93 (0.88-0.98)
2-methoxy estradiol	0.75 (0.61-0.97)
4-methoxy estrone	0.51 (0.31-1.46)
4-methoxy estradiol	0.81 (0.71-0.96)

For most of the hormones, the reproducibility of measurements was higher than 0.81, indicating a good reproducibility of the method within the range of post-menopausal women from the DOM cohort. However, for some hormones (particularly 2-methoxy estradiol and 4-methoxy estrone), the

reproducibility of measurements was less good. The latter was probably due to the very low levels of these steroids in the DOM samples (in about 50% of the samples, they were found to be under the detection limit also with our method). It was therefore decided to continue the analyses of estrogen metabolites in duplicate, to improve the measurements of exposure. As a consequence, the time required for running the analyses was doubled.

***Task 4. Extraction of DNA from cell pellets; shipment of the extracted DNA to the IARC for measurement of genetic variants.***

This task was postponed, because of the problems in the urinary hormone measurements, as mentioned above under the description of *Task 3*. In terms of priority, the first objective of this project was to examine the relationships of urinary levels of estrogen metabolites with breast cancer risk. This was also the component for which the largest part of our budget was planned. The measurement of DNA polymorphisms would have been relevant particularly with respect to statistical analyses relating the polymorphisms simultaneously to urinary levels of estrogen metabolites, as well as to breast cancer risk. However, as the first objective of our project – i.e., the hormone measurements – was subject to serious delays and technical problems, we eventually decided to focus our efforts, and remaining funds, to solving the issue of urinary hormone measurements (see description for *Task 3*, above).

***Task 5. Preparation of an exhaustive catalog of polymorphisms in the CYP1A1, CYP1B1, CYP3A4 and COMT genes by searching the literature, and by DHPLC analysis. Complete genotyping of all 300 breast cancer cases and 300 controls.***

A catalogue of polymorphisms was prepared. We did not perform genotyping, however, for the reasons explained above (see under *Task 4*).

***Task 6. Statistical analysis of the nested case-control study on urinary estrogen metabolites and breast cancer risk, and writing of reports.***

Until September 2006, measurements on samples from 290 subjects have been performed in duplicate (145 cases and 145 matched controls). Although these numbers still fall seriously short of our original study objective of 300 cancer cases and 300 control subjects, this number was sufficient for a statistical evaluation of study results. Mean levels of estrogen metabolites are shown in **Table 2**.



**Table 2. Geometric means (95% confidence intervals) of estrogens and estrogen metabolites in 145 breast cancer cases and 145 control subjects from the DOM cohort, by case/control status.**

<i>Hormones (ng/ml)</i>	<i>Cases</i>	<i>Controls</i>	<i>P diff*</i>
E1	2324.77 (2012.42-2685.60)	2119.28 (1834.54-2448.21)	0.37
E2	614.21 (449.58-839.12)	620.51 (454.19-847.73)	0.86
E3	3262.0 (2217.89-4797.66)	2524.64 (1720.75-3704.08)	0.24
16OHE1	247.04 (172.85-353.07)	330.35 (230.68-473.09)	0.19
2OHE1	2608.51 (1982.29-3432.56)	1632.37 (1249.0-2143.69)	0.02
2OHE2	302.34 (193.86-471.53)	264.24 (168.98-413.18)	0.72
4OHE1	49.95 (27.16-91.89)	54.17 (29.72-98.74)	1.00
4OHE2	1607.95 (1008.36-2564.07)	1206.79 (752.0-1936.63)	0.11
2OMeE1	496.27 (360.07-683.99)	319.08 (232.06-438.74)	0.03
2OMeE2	13.09 (7.83-21.9)	13.97 (8.35-23.36)	0.90
4OMeE1	10.12 (6.31-16.23)	7.89 (4.90-12.70)	0.24
4OMeE2	29.35 (16.45-52.35)	21.61 (12.22-38.23)	0.21

\* pairwise t-test.

These results show significantly higher urinary levels of both 2-hydroxy-estrone and 2-O-methoxy-estrone, among breast cancer cases compared to controls.

Spearman's correlation coefficients between hormones, BMI and age are shown in **Tables 3 and 4**, below

**Table 3. Spearman's correlation coefficients (adjusted for case-control status, batch, and age) between hormone concentrations and BMI, in 290 case- and control subjects from the DOM cohort.**

<b>Hormone</b>	E1	E2	E3	16OHE	2OH E	2OH E	4OH E	4OH E	2OMeE	2OMeE	4OMeE	4OMeE
E2	0.50											
E3	0.21#	0.45*										
16OHE1	0.05	0.63*	0.10									
2OHE1	0.48*	0.28#	0.02	-0.11								
2OHE2	0.18	-0.05	0.07	0.05	0.34~							

Hormone	E1	E2	E3	16OHE	2OH E	2OH E	4OH E	4OH E	2OMeE	2OMeE	4OMeE	4OMeE
4OHE1	0.51*	0.17	0.14	-0.12	0.35~	0.26~						
4OHE2	0.11	0.15	0.09	-0.15	0.15	-0.15	0.19					
2OMeE1	0.58*	0.35~	-0.03	-0.14	0.77*	0.37~	0.30~	0.08				
2OMeE2	0.20#	0.21	0.13	0.06	0.08	0.24#	0.13	0.02	0.22#			
4OMeE1	0.24#	-0.01	-0.05	-0.04	0.15	-0.11	0.28~	0.17	0.13	0.01		
4OMeE2	-0.06	0.10	0.08	-0.17	0.03	-0.16	0.02	0.24#	0.05	0.03	0.12	
BMI	0.37~	0.19	0.29~	0.02	0.06	0.03	0.09	0.13	0.15	0.16	0.12	0.08

# P<0.05; ~ P<0.01 ; \* P<0.0001

**Table 4. Spearman's correlation coefficients (adjusted for case-control status, batch, and age) between the ratios of hormone concentrations and BMI (round 1) for 290 case- and control subjects from the DOM cohort**

Hormone	2OH/ 16OHE1	2OH E2/ E3	2OH/ 4OH E1	2OH/ 4OH E2	4OH/ 16OH E1	4OH/ E3	2OMe/ 2OH E1	2OMe/ 2OH E2	4OMe/ 4OH E1	4OMe/ 4OH E2
2OHE1/16OHE1	0.15									
2OH/4OHE1	0.24#	0.04								
2OH/4OHE2	0.01	0.75*	-0.01							
4OH/16OH E1	0.72*	0.10	-0.49*	0.01						
4OHE2/E3	0.26#	0.36~	0.08	-0.35~	0.16					
2OMe/2OH E1	-0.14	0.10	-0.17	0.12	-0.02	0.02				
2OMe/2OH E2	-0.19	-0.62*	-0.08	-0.63*	-0.12	-0.06	0.11			
4OMe/4OH E1	-0.10	-0.16	0.44*	-0.27#	-0.41*	0.15	0.05	0.21#		
4OMe/4OH E2	-0.01	-0.07	0.04	-0.03	-0.05	-0.09	0.01	0.13	0.13	
BMI	0.02	-0.16	-0.04	-0.06	0.05	-0.15	0.14	0.09	0.03	-0.02

# P<0.05; ~ P<0.01 ; \* P<0.0001

Results of conditional logistic regression analyses on this first set of data (on continuous variables) are presented in **Table 5**. Urinary concentrations of 2hydroxy estrone, 2methoxy estrone and the ratio between 2hydroxy estrone/16 alpha hydroxy estrone are associated with an increase in breast cancer risk, while no association is observed for any other hormones or ratios of hormones. However, these are very preliminary analyses on a first set of data, they need to be confirmed by the analyses on the whole cohort. Since creatinine analyses on these urine samples have not been completed yet (the results of these analyses are pending), the results on absolute levels of hormones should be interpreted carefully. The delay in analyses is related to the fact that

estrogen metabolites analyses are done on mixed aliquots of samples from different collections over-time, so samples are thawed and mixed together on the day of analyses, to avoid repeated thawing and freezing cycles. Creatinine analyses will be performed only when all samples will be mixed, at the end of the GC/MS analyses.

**Table 5. Relative risk of breast cancer (95% confidence intervals) by hormone level (on a continuous scale), adjusted for BMI.**

<i>Hormone</i>	<i>OR</i>	<i>P trend</i>
E1	1.64 (0.80-3.39)	0.18
E2	1.04 (0.77-1.40)	0.80
E3	1.17 (0.92-1.48)	0.21
16OHE1	0.86 (0.70-1.05)	0.15
<b>2OHE1</b>	<b>1.79 (1.03-3.13)</b>	<b>0.04</b>
2OHE2	1.03 (0.87-1.20)	0.76
4OHE1	1.01 (0.77-1.32)	0.95
4OHE2	1.35 (0.94-1.94)	0.10
<b>2OMeE1</b>	<b>1.56 (1.05-2.32)</b>	<b>0.03</b>
2OMeE2	1.01 (0.79-1.27)	0.96
4OMeE1	1.17 (0.93-1.48)	0.17
4OMeE2	1.14 (0.93-1.41)	0.21
<b>2OHE1/ 16OHE1</b>	<b>1.37 (1.04-1.80)</b>	<b>0.03</b>

**Task 7. Statistical analysis of relationships of genotypes with urinary estrogen metabolites and breast cancer risk. (Months 12-24);**

For the reasons explained above (see description of problems under “Task 4”), we did not perform DNA extractions, and did not perform the genotyping.

## KEY RESEARCH ACCOMPLISHMENTS

Over the past four years, we selected 324 cases and 324 matched controls from the DOM cohort. Urine samples were selected for analyses. After a substantial initial delay, we started the measurements of estrogen metabolites in the DOM samples in the laboratory for hormone

analyses at IARC. Until September 2006, we have also been able to perform 50% of the analyses that were foreseen to complete the study

## **REPORTABLE OUTCOMES**

There are no reportable outcomes so far; however, we are continuing the hormone assays (using internal IARC funds) to complete the urinary hormone measurements of all breast cancer cases and controls, and plan to write one scientific report based on the study results.

## **CONCLUSIONS**

In conclusion, due to a substantial delay occurred during the first two years of the project, the current project has not reached completion. However, estrogen metabolites from urine samples of about 50% of the subjects included in the study have already been analyzed, and we plan to continue the urinary hormone measurements during 2007, using internal IARC funds. Statistical analyses will then be run, and we plan to prepare one manuscript on the relationship of estrogen metabolites in urine and breast cancer risk in post-menopausal women will be written, corresponding to our principal study objective.